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Evaluation of Argyrophilic Nucleolar Organizer Region and Proliferating Cell Nuclear Antigen in Colorectal Cancer

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Background and Objectives: Information on cellular proliferation is gaining importance for predicting prognosis in several cancers. To clarify the clinicopathological significance of argyrophilic nucleolar organizer region (AgNOR), proliferating cell nuclear antigen (PCNA), and DNA ploidy pattern, we studied their correlations with clinicopathological factors in colorectal cancer.
Methods: Fifty-two patients with colorectal cancer were examined by AgNOR staining, immunohistochemical study of PCNA expression, and DNA flow cytometry.
Results: The AgNOR score and the PCNA labeling rate (PCNA LR) were significantly higher in patients with deep invasion ($P = 0.0072$, $P = 0.0355$), liver metastasis ($P = 0.0022$, $P = 0.0001$), and Dukes D classification ($P = 0.0002$, $P = 0.0001$) than in patients without these factors. In patients with high AgNOR score (>3.83) or with high PCNA LR (>48.8), prognosis was significantly worse ($P = 0.0002$, $P = 0.0123$) than in those with low AgNOR score (<3.83) or in those with low PCNA LR (<48.8), respectively. No significant association was observed between AgNOR score and PCNA LR. Combined analysis revealed that the survival curve for patients with high AgNOR score and high PCNA LR was significantly lower ($P = 0.0156$) than that for patients with high AgNOR score and low PCNA LR. There was no significant correlation between DNA ploidy pattern and clinicopathological findings.
Conclusions: PCNA LR and AgNOR score were correlated not only with local progression but also with metastasis. Their determination provided useful prognostic information, and these parameters are probably independent. Their simultaneous determination was useful for accurate evaluation of prognosis. The value of DNA ploidy pattern was uncertain.
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KEY WORDS: DNA ploidy; colorectal cancer; prognosis; silver stain; gold toning

INTRODUCTION

In addition to the conventional pathological parameters, several experimental techniques have been shown to provide prognostic value in several human malignancies [1,2]. Recently, information on proliferative kinetics such as DNA flow cytometry or cell proliferation has been introduced and is gaining importance for predicting the course of disease [3–6].

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Nucleolar organizer regions (NORs) are loops of DNA in the nuclei of cells which code for ribosomal RNA and contribute to the regulation of cellular protein synthesis [7]. The argyrophilic nucleolar organizer region (AgNOR) method stains NOR-associated proteins [8] and the AgNOR count of a tumor is, therefore, thought to correlate with the proliferation rate.

Proliferating cell nuclear antigen (PCNA), a 36 kD protein, was identified as an auxiliary protein of the DNA polymerase delta [9,10]. Recently, PCNA has been available in immunohistochemical studies of cell proliferation because its expression is closely linked to the cell cycle [11] and would be expected to provide useful information concerning the malignant potential of tumors [12].

Nuclear DNA content of tumor cells can be rapidly measured by flow cytometry. Many studies have reported the prognostic influence of DNA content. However, the results have varied greatly and have been a matter of controversy [3,13–17].

The aim of this study was to clarify the clinicopathological significance of these techniques in evaluating the grade of malignancy in colorectal cancer.

MATERIALS AND METHODS

Fifty-two patients (36 with colonic carcinoma and 16 with rectal carcinoma), who were diagnosed and treated at the First Department of Surgery, Kobe University Hospital (Kobe, Japan), from July 1991 to December 1993, were examined in this study. All of the patients underwent resection of the tumor combined with lymph node dissection, and none of them received preoperative chemotherapy or radiation therapy. The resected specimens were fixed in 10% buffered formalin and processed onto paraffin wax. The specimens were examined both macroscopically and histologically according to the Japanese Classification of Colorectal Carcinoma [18].

AgNOR Staining

Sections, 3 μm thick, from paraffin blocks of the specimens were dewaxed in xylene, rehydrated in a series of graded ethanols, and washed in water. They were stained for AgNORs as described by Crocker and Nar [19] as follows in brief. The sections were incubated in a solution consisting of 1 volume of 2% gelatin in 1% formic acid and 2 volumes of 50% silver nitrate at 40°C for 40 min. After washing in distilled water, the sections were toned in 0.2% gold chloride solution for 10 min [20]. The sections were then rinsed in a steady stream of water and fixed in 5% sodium thiosulfate for 1 min. After washing thoroughly with distilled water, methyl-green counterstain was applied for 1 min to each section, which was then rinsed in distilled water, dehydrated, and mounted in a routine fashion.

AgNORs were visualized as black dots (Fig. 1). A total of 200 nuclei from each case was counted under a microscope at a magnification of ×400 in the advancing margin of the tumor, and the mean number of AgNORs per nucleus was determined as the AgNOR score.

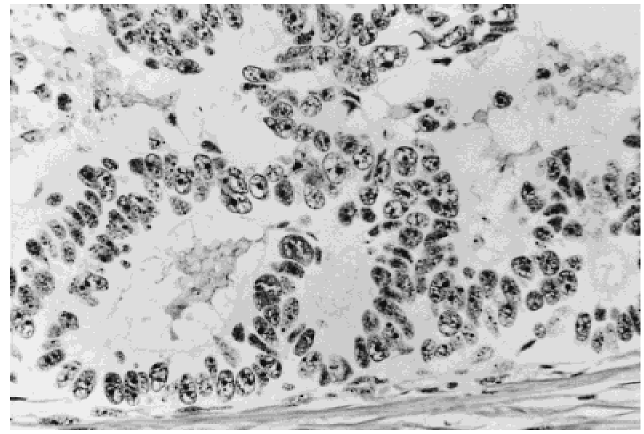

Fig. 1. AgNOR staining of colorectal cancer. Black dots were visualized within the nuclei (×400).

Immunohistochemical Study of PCNA Expression

Sections (4 μm) were cut and mounted on poly-L-lysine-coated glass slides. The slides were dewaxed and rehydrated, and then the PCNA staining was performed according to the method described by Nakae et al. [21] and Nakamura et al. [22] as follows. Endogenous peroxide activity was blocked by 3% hydrogen peroxide. Nonspecific binding of the second antibody was diminished by preincubation with normal bovine serum. PC10 (Dakopatts, Glostrup, Denmark), a mouse monoclonal anti-PCNA antibody [23], was diluted 1:20 in Tris-buffered saline (0.05 M, pH 7.6) and reacted with tissue specimens overnight at 4°C. The sections were then incubated with a biotinylated goat antimouse secondary antibody for 10 min at room temperature. Slides were reacted with peroxidase-labeled streptavidin for 10 min and 3-amino-9-ethylcarbazole (AEC) was used as chromogen. Finally, the slides were counterstained with hematoxylin.

Positive staining of PCNA was localized in the nuclei and appeared diffuse or granular, whereas the cytoplasms and cell membranes remained unstained (Fig. 2). Nuclei from a minimum of 1,000 tumor cells in the advancing margin of the tumor were microscopically counted in each sample. The PCNA labeling rate (PCNA LR) was determined as the percentage of positive cell nuclei. To ensure consistency of PCNA staining between batches, a known positive control colonic carcinoma was included in each round. Substitution of the primary antibody by Tris-buffered saline was employed as a negative control.

Both AgNORs and immunostained nuclei with PC10 were counted by a single observer without knowledge of the clinicopathological data.

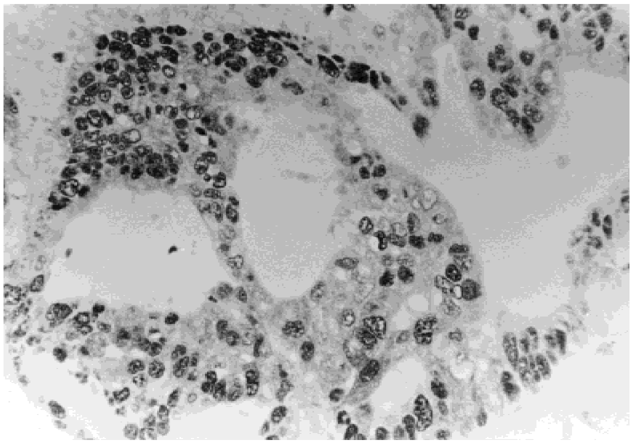

Fig. 2. Immunohistochemical staining of PCNA in colorectal cancer. Positive cells were identified by dark nuclear staining (×400).

DNA Flow Cytometry

Flow cytometric analysis of nuclear DNA was performed in all 52 cases on paraffin-embedded tumor materials obtained from 28 cases and on frozen tumor materials from 24 cases.

Using paraffin-embedded tissue, 40-μm-thick sections were made and dewaxed in xylene and rehydrated through graded ethanols to distilled water. Then the specimen was incubated in 0.25% trypsin in citrate buffer overnight at 37°C. The cells were filtered through a 40 μm nylon mesh and stained according to the method described by Vindeløv et al. [24]. After infiltration through a 40 μm nylon mesh, cellular DNA content was measured with a flow cytometer (FACScan).

In the case of frozen materials, samples were defrosted and minced into small pieces of 1 mm, followed by filtration through a nylon mesh with pore size 40 μm. They were incubated with 0.1% Triton X-100 containing 0.1% RNAase. After filtration through a 40 μm nylon mesh, the cell suspensions were stained with propidium iodide (50 μg/ml) and filtered through a 40 μm nylon mesh. Thereafter, DNA ploidy was analyzed by a flow cytometer (FACScan, Becton Dickinson, San Jose, California, USA).

Tumor samples were defined as ''diploid'' when they had a single peak and their DNA index (peak DNA value of tumor cells/peak DNA value of normal epithelial cells) was within a range of 0.95 and 1.05. DNA histograms other than diploid were classified as ''aneuploid'' in this study. Nuclear DNA ploidy patterns of three tumors could not be assigned because of cellular destruction.

Throughout this report, the Japanese Classification of Colorectal Carcinoma [18] was used for the terms and clinicopathological findings. Statistical analyses were performed by the Student *t*-test and the chi-square test or the Fisher exact probability calculation method. The correlation between PCNA LR and AgNOR score was studied using linear regression. Survival curves and rates were estimated by the Kaplan-Meier method, and the outcomes of different groups of patients were compared by the generalized Wilcoxon test. Statistical significance was defined as $P < 0.05$.

TABLE I. AgNOR Scores in Cancer and Normal Mucosa

	No. of cases	AgNOR score (mean ± SD)[a]	*P*
Cancer	43	3.84 ± 0.32	<0.0001
Normal mucosa	43	2.68 ± 0.23	

[a]AgNOR scores are per nucleus.

RESULTS

Clinicopathological Findings, AgNOR Score, and Survival

AgNOR scores of 52 colorectal cancers ranged from 3.17 to 4.55 [3.83 ± 0.30, mean ± standard deviation (SD)]. For 43 cases, AgNOR scores of both normal mucosa and cancer were counted. The mean AgNOR scores of colorectal cancer were higher than those of normal colorectal mucosa ($P < 0.0001$) (Table I). The relationship between the clinicopathological findings and the AgNOR score of all 52 cases is summarized in Table II. No significant difference was found in the correlations between AgNOR score and age ($P = 0.5935$), tumor size ($P = 0.3486$), histological type ($P = 0.6868$), lymph node metastasis ($P = 0.1183$), or venous invasion ($P = 0.1505$). On the other hand, statistically significant differences ($P < 0.05$) were present in depth of invasion, peritoneal metastasis, liver metastasis, and histological stage classification.

With regard to the depth of invasion, the mean AgNOR score of tumors with serosal invasion was significantly higher ($P = 0.0072$) than that of tumors without serosal invasion. The mean AgNOR score was significantly higher in patients with peritoneal metastisis ($P = 0.0424$) or liver metastasis ($P = 0.0022$) than in patients without metastasis, respectively. In addition, concerning the lymphatic invasion, the mean AgNOR score of tumors with moderate or severe invasion tended to be higher ($P = 0.0684$) than that of tumors with no or minimal invasion. As for the histological stage classification, the mean AgNOR score of patients in Dukes D was significantly higher ($P = 0.0002$) than that of patients in Dukes A, Dukes B, or Dukes C (Table II).

No significant relationship was observed between AgNOR score and DNA ploidy pattern ($P = 0.3231$) (Table II).

The 52 tumors were divided into 2 groups by the mean value of AgNOR score: tumors with AgNOR scores of >3.83 were designated as the high AgNOR group and those with AgNOR scores of <3.83 were defined as the

TABLE II. Correlation of AgNOR Score and PCNA LR With Clinicopathological Findings

Variables	n	AgNOR score (mean ± SD)	*P*	PCNA LR (mean ± SD)	*P*
Age					
<65 years	24	3.81 ± 0.32	0.5935	51.8 ± 12.4	0.1361
≧65 years	28	3.86 ± 0.29		46.3 ± 13.7	
Tumor size					
<50 mm	21	3.79 ± 0.34	0.3486	45.2 ± 13.8	0.1130
≧50 mm	31	3.87 ± 0.28		51.2 ± 12.6	
Histological type[a]					
Well, Mod	46	3.83 ± 0.28	0.6868	48.0 ± 13.4	0.2610
Poor, Muc	6	3.88 ± 0.47		54.6 ± 12.0	
Depth of invasion[b]					
~ss a_1	25	3.72 ± 0.29	0.0072	44.8 ± 13.4	0.0355
se a_2~	27	3.94 ± 0.29		52.5 ± 12.2	
Peritoneal metastasis					
Negative	50	3.82 ± 0.30	0.0424	48.2 ± 13.2	0.1252
Positive	2	4.26 ± 0.28		63.0 ± 25.9	
Liver metastasis					
Negative	41	3.77 ± 0.30	0.0022	44.8 ± 11.8	0.0001
Positive	11	4.08 ± 0.18		63.8 ± 56.1	
Lymph node metastasis					
Negative	26	3.77 ± 0.21	0.1183	45.9 ± 12.4	0.1109
Positive	26	3.90 ± 0.37		51.7 ± 13.7	
Lymphatic invasion					
None or minimal	34	3.78 ± 0.29	0.0684	46.2 ± 13.8	0.0528
Moderate or severe	18	3.94 ± 0.32		53.7 ± 10.9	
Venous invasion					
None or minimal	32	3.79 ± 0.28	0.1505	46.9 ± 14.4	0.1837
Moderate or severe	20	3.91 ± 0.33		51.9 ± 10.9	
Dukes classification					
A	11	3.74 ± 0.20		40.6 ± 13.4	
B	14	3.76 ± 0.20 3.75 ± 0.29		49.0 ± 10.5 44.3 ± 11.9	
C	14	3.75 ± 0.42	0.0002	42.4 ± 11.1	0.0001
D	13	4.09 ± 0.17		62.4 ± 6.1	
DNA ploidy pattern					
Diploid	23	3.88 ± 0.31	0.3231	47.1 ± 15.4	0.2812
Aneuploid	26	3.79 ± 0.31		51.0 ± 9.5	

[a]Well = well-differentiated adenocarcinoma; Mod = moderately differentiated adenocarcinoma; Poor = poorly differentiated adenocarcinoma; Muc = mucinous carcinoma.
[b]~ss a_1 = sm, mp, ss a_1; se a_2~ = se a_2, si ai.

low AgNOR group. Figure 3 shows the overall survival curves of these groups. The prognosis of the high AgNOR group was significantly worse than that of the low AgNOR group ($P = 0.0002$).

Clinicopathological Findings, PCNA LR, and Survival

PCNA LR of 52 colorectal cancers ranged from 23.8 to 75.4 (48.8 ± 13.3, mean ± SD). As shown in Table II, statistically significant differences ($P < 0.05$) existed with respect to depth of invasion, liver metastasis, and histological stage classification.

In cases with the depth of invasion, the mean PCNA LR of tumors with serosal invasion was significantly higher ($P = 0.0355$) than that of tumors without serosal invasion. The mean PCNA LR was significantly higher ($P = 0.0001$) in patients with liver metastasis than in patients without metastasis. In addition, regarding the lymphatic invasion, the mean PCNA LR of tumors with moderate or severe invasion tended to be higher ($P = 0.0528$) than that of tumors with no or minimal invasion. As far as the Dukes classification was concerned, the mean PCNA LR of patients in Dukes D was significantly higher ($P = 0.0001$) than that of patients in Dukes A, Dukes B, or Dukes C (Table II).

There was no significant correlation between PCNA LR and DNA ploidy pattern ($P = 0.2812$) (Table II).

The 52 tumors were classified into 2 groups by the mean value of PCNA LR: the high PCNA group for which the PCNA LR was >48.8 and the low PCNA group for which the PCNA LR was <48.8. The overall survival curves of these groups are depicted in Figure 4. The prognosis of the high PCNA group was significantly worse than that of the low PCNA group ($P = 0.0123$).

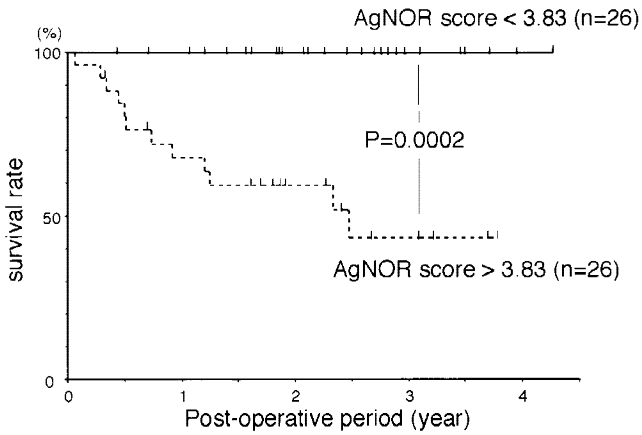

Fig. 3. Survival curves according to AgNOR score. The prognosis of the high AgNOR group was significantly worse than that of the low AgNOR group ($P = 0.0002$).

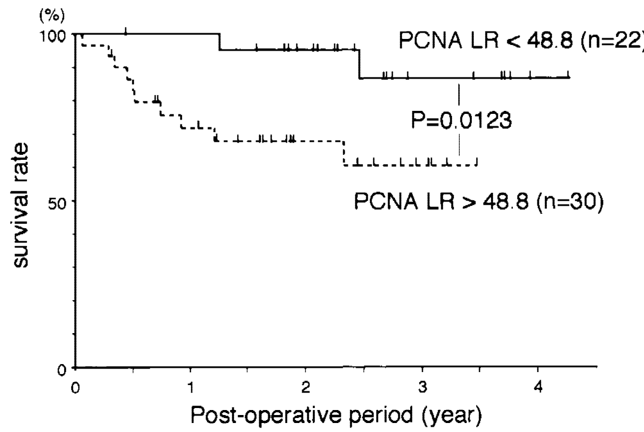

Fig. 4. Survival curves according to PCNA LR. The prognosis of the high PCNA group was significantly worse than that of the low PCNA group ($P = 0.0123$).

Relationship Between AgNOR Score and PCNA LR

Figure 5 is a scattergram showing the correlation between PCNA LR and AgNOR score. There was no linear relationship between PCNA LR and AgNOR score ($r = 0.24$, $P = 0.087$).

Combined Analysis of AgNOR Score and PCNA LR

Because a significant difference in the prognosis was found between the low and high PCNA groups, the effect of PCNA LR on the prognosis of the high AgNOR group was analyzed, For the high AgNOR group, the prognosis of the high PCNA group was significantly worse than that of the low PCNA group ($P = 0.0156$). In addition, the survival curves of these two groups were significantly worse ($P = 0.0001$, $P = 0.0302$) than those of the low AgNOR group (Fig. 6).

Clinicopathological Findings, DNA Ploidy Pattern, and Survival

Twenty-three patients had tumors with diploid DNA ploidy patterns, whereas 26 were classified as aneuploid. Table III shows a correlation between tumor ploidy and clinicopathological findings. Ploidy pattern appeared to have no relationship to any of the clinicopathological findings ($P > 0.1$). The Kaplan-Meier curves for the overall survival of patients with diploid and aneuploid tumors are given in Figure 7. There was a weak trend toward a somewhat poorer survival for patients with diploid compared with aneuploid tumors. However, the survival rates for the two ploidy groups did not differ significantly ($P = 0.1104$).

DISCUSSION

Although various methods for estimating the proliferative activity of tumor cells have been introduced recently

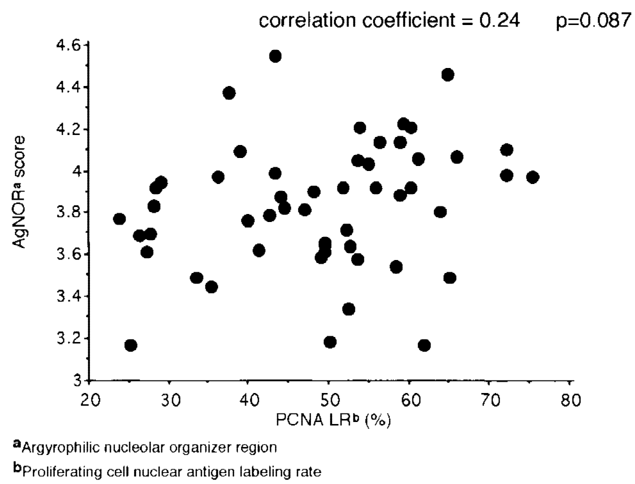

Fig. 5. Relationship between AgNOR score and PCNA LR. There was no linear relationship between AgNOR score and PCNA LR ($r = 0.24$, $P = 0.087$).

[25–27], the AgNOR method and PCNA immunostaining using PC10 [23], which is an anti-PCNA antibody and does not require fresh tissue, and the analysis of DNA ploidy pattern have the advantage of applicability to formalin-fixed tissues. Therefore, these methods make it possible to study archival material for which survival data are already available. Concerning the silver colloid technique for staining NORs, Delahunt et al. [20] reported that the application of gold toning had provided improved definition of AgNORs, consequently we used this modification.

Since the reports of Crocker and Nar [19] were published, the AgNOR technique has been applied to metaphase chromosome spreads and has been used to identify atypical chromosomes in certain malignancies [28–32]. Furthermore, some investigators show the prognostic significance of AgNOR measurement [29,30], while others

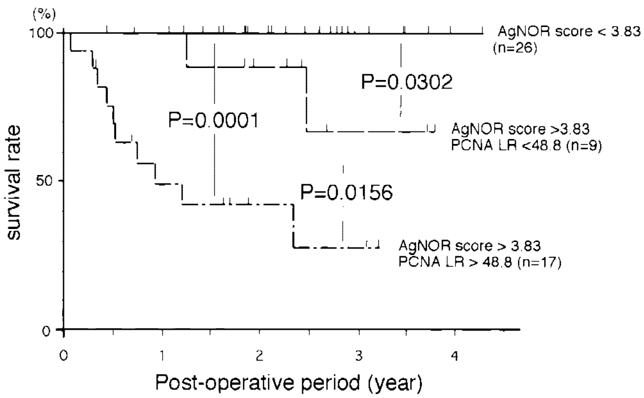

Fig. 6. Survival curves according to AgNOR score and PCNA LR. For the high AgNOR group, the prognosis of the high PCNA group was significantly worse than that of the low AgNOR group ($P = 0.0156$). In addition, the survival curves of these two groups were significantly worse ($P = 0.0001$; $P < 0.0302$) than those of the low AgNOR group.

fail to demonstrate a relationship between AgNOR count and clinical outcome [32]. Kakeji et al. [28] reported that gastric cancers characterized by lymph node metastasis and lymphatic or venous invasion had a significantly higher AgNOR count than those without such metastatic behaviors. As for colorectal tumors, Chen et al. [31] observed that the interphase AgNOR count permitted a significant difference between malignant and benign or normal mucosa and that the mean number of AgNOR dots in well-differentiated adenocarcinoma was significantly lower than that of moderately or poorly differentiated adenocarcinoma. In addition, Joyce et al. [29] and Yamaguchi et al. [30] reported that the AgNOR score provided useful prognostic information. However, Adachi et al. [32] described that the AgNOR count did not correlate with clinicopathological parameters and the survival curve was not affected by the AgNOR count. Al-Sheneber et al. [33] described that the PCNA LR might be helpful as a predictor of survival.

In the present study, the AgNOR count was connected not only with the depth of invasion, but also with peritoneal metastasis and liver metastasis. Regarding the PCNA LR, similar correlations with clinicopathological findings were observed in our study. Particularly with liver metastasis, which is one of the most important prognostic factors for colorectal cancer patients, both proliferative markers have a close correlation. These data suggest that the proliferative activity of the invasive tumor margin is associated not only with local progression, but also with metastasis. In addition, these two parameters seem to be in close correlation with the prognosis of colorectal cancer. These results indicate that colorectal cancer showing a high AgNOR count or a high PCNA LR may have a potential risk of liver metastasis.

Several researchers have reported the significant correlation between two parameters indicating proliferative activity [28,34–37], but there have been no reports on the relationship between AgNOR count and PCNA LR in colorectal cancer. No significant correlation between AgNOR count and PCNA LR was found in the present study. Thus, these two parameters are probably independent and may evaluate different aspects of proliferation.

The combined analysis of AgNOR score and PCNA LR in this study revealed that the prognosis of the high PCNA group was significantly worse than that of the low PCNA group in the high AgNOR group. This result indicates that simultaneous determination of these two parameters is more useful for accurate evaluation of prognosis of colorectal cancer patients than either determination alone.

Many authors have emphasized the prognostic influence of DNA content in colorectal cancer as measured by flow cytometry [3,13]. However, some studies could not

TABLE III. Clinicopathological Findings and DNA Ploidy Pattern

		DNA ploidy pattern		
Variables	n	Diploid	Aneuploid	P
Age				
<65 years	24	10	14	0.4687
≧65 years	25	13	12	
Diameter of tumor				
<50 mm	19	7	12	0.2597
≧50 mm	30	16	14	
Pathology[a]				
Well, Mod	43	19	24	0.3013
Poor, Muc	6	4	2	
Depth of invasion[b]				
~ss a_1	24	10	14	0.4687
se a_2~	25	13	12	
Peritoneal metastasis				
Negative	47	22	25	0.9294
Positive	2	1	1	
Liver metastasis				
Negative	39	17	22	0.3536
Positive	10	6	4	
Lymph node metastasis				
Negative	26	11	15	0.4898
Positive	23	12	11	
Lymphatic invasion				
None or minimal	32	14	18	0.5394
Moderate or severe	17	9	8	
Venous invasion				
None or minimal	30	13	17	0.5251
Moderate or severe	19	10	9	
Dukes classification				
A	11	6	5	
B	14] 37	4] 16	10] 21	
C	12	6	6	0.3627
D	12	7	5	

[a]Well = well-differentiated adenocarcinoma; Mod = Moderately differentiated adenocarcinoma; Poor = poorly differentiated adenocarcinoma; Muc = mucinous carcinoma.
[b]~ss a_1 = sm, mp, ss a_1; se a_2~ = se a_2, si ai.

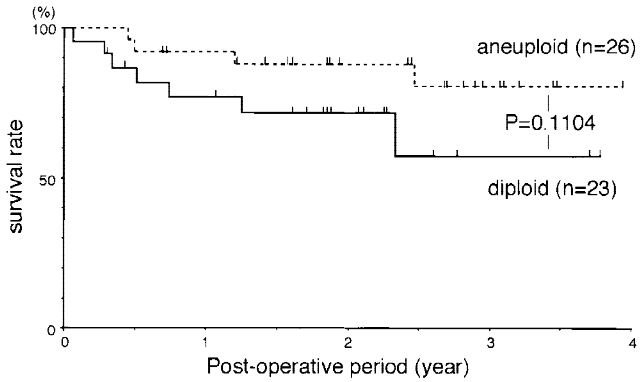

Fig. 7. Survival curves dependent on DNA content. There was a weak trend toward a somewhat poorer survival for patients with diploid compared with aneuploid tumors. However, the survival rates for the two ploidy groups did not differ significantly ($P = 0.1104$).

find it useful in estimating the outcome of patients [14–17].

In our study, there was no significant correlation of DNA ploidy pattern with clinicopathological variables and the two proliferative markers. In addition, it showed no statistically significant correlation with survival.

CONCLUSIONS

PCNA LR or AgNOR score correlated not only with local progression, but also with metastasis. Their determination provided useful prognostic information, and these parameters were probably independent. Simultaneous determination of these factors was more useful for accurate evaluation of prognosis than determination of either one by itself. The value of DNA ploidy pattern was uncertain in the prediction of prognosis.

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